Characterization of a macrophage-derived plasminogen-activator inhibitor

Similarities with placental urokinase inhibitor

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Human and mouse macrophages release a fibrinolytic inhibitor after stimulation by endotoxin *in vitro*. The released mouse inhibitor was indistinguishable in size by molecular-sieve chromatography from an intracellular form (approx. 50 kDa), and both inhibitors blocked urokinase directly as judged by a ^{125}I -plasminogen conversion assay. The intracellular inhibitor was found mostly to dissociate from ^{125}I -urokinase during sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under reduced conditions, but a dodecyl sulphate-stable complex at 65–67 kDa was observed. Because of similarities in the reported size, stability and urokinase-binding properties of a placental urokinase inhibitor, the kinetic properties of the two inhibitors were compared. Under the reaction conditions employed (37°C at pH 7.4 in the presence of 0.2% Triton X-100), the association rate constants and equilibrium dissociation constants of the two inhibitors were indistinguishable, $3 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $4 \times 10^{-10} \text{ M}$ respectively. These data show that peritoneal macrophages contain a plasminogen-activator very similar to a previously recognized placental inhibitor. Although the inhibitor appears to be a trace protein in macrophages, placental macrophages may account for the accumulation of the inhibitor in placental tissue.

Stimulated mouse and human macrophages at extravascular sites of inflammation regulate the deposition and turnover of fibrin/fibronectin by at least three mechanisms. First, the cells secrete fibronectin and synthesize and express on their cell surface the initial enzyme complex of the extrinsic pathway of coagulation (Rennard *et al.*, 1981; Chapman *et al.*, 1985). Secondly, the cells express a membrane-bound form of a urokinase-type plasminogen activator (PA) and secrete a similar enzyme in soluble form (Chapman *et al.*, 1984; Lemaire *et al.*, 1983). Thirdly, the cells, on stimulation with endotoxin, accumulate intracellularly and release extracellularly a fibrinolytic inhibitor of urokinase-type PA (Chapman *et al.*, 1982). The expression of these several factors by macrophages seems to be co-ordinately regulated to favour either fibrin/fibronectin accumulation or resorption. The deposition and organization of

fibrin/fibronectin in tissues may in turn influence the mobility and distribution of macrophages and the course of healing (Leibovich & Ross, 1975; Repesh *et al.*, 1982; Hedelin *et al.*, 1983; Chapman *et al.*, 1983). Several lines of evidence suggest that fibrin/fibronectin in tissues may serve as a provisional matrix for fibroblast proliferation and subsequent scar formation (Kwaan & Astrup, 1969; Kurkinen *et al.*, 1980; Grinnell *et al.*, 1980; Bitterman *et al.*, 1983; Holund *et al.*, 1984).

The nature of the fibrinolytic inhibitor expressed by endotoxin-stimulated macrophages is unclear. Recent observations in this laboratory indicate that human alveolar macrophages, on induction with endotoxin, express a PA inhibitor that binds ^{125}I -urokinase. Moreover, the binding pattern, as judged by autoradiography after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, is virtually indistinguishable from that of a previously recognized urokinase inhibitor from human placenta (H. A. Chapman & O. L. Stone, unpublished work). The placental inhibitor was the first PA inhibitor to be characterized in terms of size, physical stability, and interaction

Abbreviation used: PA, plasminogen activator.

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with urokinase (Kwano *et al.*, 1970). The kinetics of binding of the placental inhibitor with urokinase have been reported (Christensen *et al.*, 1982). Insufficient human macrophage PA inhibitor is available for a similar analysis. In the present study we have examined the functional properties of a partially purified preparation of PA inhibitor from endotoxin-stimulated mouse peritoneal macrophages. In particular, we have examined the physical nature and kinetics of interaction of the macrophage inhibitor with urokinase and compared the results with the properties of a placental inhibitor preparation examined under identical conditions.

Experimental

Reagents

Animal and human sera were prepared and stored as previously described (Chapman *et al.*, 1982). Human plasminogen was obtained from Helena Laboratories (Houston, TX, U.S.A.) and determined to be free of detectable plasmin in a ^{125}I -fibrin plate assay at concentrations used in the experiments. Urokinase, obtained from the American Red Cross (Washington, DC, U.S.A.), was stored in small portions at -70°C . Urokinase and plasminogen were radiolabelled with carrier-free ^{125}I (specific radioactivity 17 Ci/mg; New England Nuclear, Boston, MA, U.S.A.) by a chloramine-T method (Kaplan & Nielsen, 1979). The unbound ^{125}I was removed by molecular-sieve chromatography (Sephadex G-10) in the presence of bovine serum albumin carrier (1 mg/ml), and the labelled urokinase or plasminogen was divided into small portions and stored at -70°C . Human fibrinogen was obtained from Merck (West Point, PA, U.S.A.) and made plasminogen-free by repeated ethanol precipitation in the presence of lysine (Mosesson, 1962). ^{125}I -fibrinogen was obtained from Amersham (Chicago, IL, U.S.A.). A partially purified preparation of human placental urokinase inhibitor was obtained from α -Therapeutics (Los Angeles, CA, U.S.A.). Sodium dodecyl sulphate was obtained from Gallard-Schlesinger (New York, NY, U.S.A.). Endotoxin, a Westphal preparation of *Salmonella enteritidis* (Sigma Chemical Co., St. Louis, MO, U.S.A.), was used without further purification. Benzyloxycarbonyl-glycyl-glycyl-arginine 4-aminomethylcoumarinamide (Cbz-Gly-Gly-Arg-Amc; Enzyme Systems Products, Livermore, CA, U.S.A.), a previously studied substrate for urokinase, was used for fluorimetric assays of urokinase (Zimmerman *et al.*, 1978; Chapman *et al.*, 1982).

Cell culture and PA inhibitor preparation

C₃H/HeN female mice obtained from the

Frederick Cancer Research Center Animal Facility (Frederick, MD, U.S.A.) were injected with 1 ml of thioglycollate medium (Difco, Detroit, MI, U.S.A.) intraperitoneally 3 days before use. Cells were harvested, counted and adhered to 90 mm glass Petri dishes. After 1 h at 37°C in a humidified incubator with 5% CO₂ in air, cell monolayers were washed three times in phosphate-buffered saline (NaCl/sodium phosphate buffer, pH 7.4) and then incubated for 18 h in serum-free tissue-culture medium containing 100 ng of endotoxin (Westphal; Sigma Chemical Co.)/ml. The basic tissue-culture medium used for all experiments was Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, NY, U.S.A.). Cell monolayers (240×10^6 total cells) were washed again after culture and lysed in phosphate-buffered saline with 0.2% Triton X-100 for 20 min at 37°C . The lysate was clarified by centrifugation and then subjected to molecular-sieve chromatography as previously described (Chapman *et al.*, 1982). The inhibitor-rich fraction was dialysed extensively against water (pH 7) and then freeze-dried and stored at 4°C until use. The average total protein in three such preparations was 1.36 mg.

In some experiments, 25×10^6 cells were made adherent to 25 cm² plastic culture flasks (Falcon). The washed monolayers were then incubated in 1 ml of 1% (v/v) acid-treated plasminogen-free dog serum with or without endotoxin (100 ng/ml) for 18 h. The cell-free supernatants were used either as a source of soluble PA (endotoxin-free) or as a PA-inhibitor-rich medium (endotoxin-supplemented). Cell lysates (10^6 cells/100 μl of 0.2% Triton/phosphate-buffered saline) were tested similarly.

Assays of PA or PA inhibitor

Fibrinolytic activity or inhibitor activity was first assayed in ^{125}I -fibrin-coated tissue-culture wells as previously described (Chapman *et al.*, 1982). Briefly, the cell-free supernatants or lysates (100 μl) were assayed for inhibitor by mixing with 100 μl of urokinase (0.5 unit) followed by incubation in ^{125}I -fibrin-coated wells with plasminogen (8 $\mu\text{g}/\text{ml}$) for 4 h at 37°C . Degraded fibrin was assayed by β -scintillation counting. Inhibitor activity is expressed as percentage inhibition of urokinase activity in control wells containing unconditioned media and represents a mean of triplicate determinations. The assays were generally done concurrently with ^{125}I -plasminogen conversion assays, described below.

The kinetic analysis of the interaction of the PA inhibitor with urokinase was done by fluorimetry basically as described by Christensen and co-workers but with some modifications (Christensen *et al.*, 1982). The interaction of the inhibitor with urokinase was studied at 37°C in phosphate-

buffered saline containing 0.2% Triton X-100. The Triton was included because the partially purified inhibitor preparation contained small amounts of Triton and this critically influenced the reaction velocity. Triton, as reported by other investigators, appreciably increased the reaction rates (Unkeless *et al.*, 1974). By performing all assays in 0.2% Triton, this variable could be eliminated. The molarity of the urokinase preparation was first determined by five separate active-site titrations with *p*-nitrophenyl guanidinobenzoate. The urokinase preparation was found to contain 0.12 pmol/unit. Small portions were stored at -70°C at 250 units/ml. The basic reaction conditions for subsequent experiments were urokinase (5.6–11.2 nM) and various amounts (2–30 μl) of inhibitor (1.36 mg/ml) in a total volume of 500 μl of phosphate-buffered saline containing 0.2% Triton X-100. After various times of incubation at 37°C , the urokinase substrate Cbz-Gly-Gly-Arg-Amc was added in 10 μl to a final concentration of 0.1 mM, and the initial reaction rate was determined at 37°C in a Perkin-Elmer spectrofluorimeter interfaced with an X-Y recorder. The instrument settings were as previously described (Chapman *et al.*, 1982). In some experiments the substrate concentration was increased to 1.0 mM final concentration. Preliminary data showed that no enzyme activity towards the urokinase substrate was measurable in the PA inhibitor preparation. However, it should be noted that the experiments cannot be done with crude lysates of the cells because the lysates contain PA as well as the inhibitor. We have reported the macrophage membrane PA to be relatively resistant to the PA inhibitor (Chapman *et al.*, 1982). Presumably much or most of the inhibitor is membrane-bound in the lysate and fractionates at a different molecular mass from that of the inhibitor during chromatography because the inhibitor-rich fraction is devoid of PA activity.

The molarity of the PA inhibitor in the preparations tested was determined by titration of urokinase with various microlitre amounts of inhibitor. The enzyme and inhibitor were allowed to interact at 37°C for 60 min before analysis. The fractional urokinase activity (E/E_0) was observed to decline in linear fashion with increasing microlitre amounts of inhibitor until E/E_0 was not more than 0.35. Under these conditions, if the enzyme concentration is known, the inhibitor concentration can be calculated from the slope of the line (Christensen *et al.*, 1982). By this technique, the three preparations used for analysis in this study, at total protein concentrations of 1.4 mg/ml, were 170 nM, 112 nM and 145 nM in PA inhibitor.

In experiments with the placental inhibitor

instead of the macrophage inhibitor, the freeze-dried placental inhibitor was also reconstituted in 0.2% Triton X-100/phosphate-buffered saline and the molarity determined by titration of urokinase as described above.

Autoradiography

We examined the fibrinolytic inhibitors by two autoradiographic techniques. Purified human plasminogen was iodinated and the inhibitor tested for its capability to block conversion of ^{125}I -plasminogen into ^{125}I -plasmin. The assay was performed as described by Loskutoff & Edgington (1981). Briefly, 5 μl of ^{125}I -plasminogen, 1.25 units of urokinase (5 μl), 6.25 units of Trasylol and 15 μl of the test sample were made 0.2% with respect to Triton X-100, mixed, and incubated for 4 h at 37°C . The mixture was then diluted with 25 μl of 3% (w/v) sodium dodecyl sulphate/10% (v/v) glycerol/62.5 mM-Tris/HCl buffer, pH 6.3, containing 2-mercaptoethanol (10%, v/v), boiled for 2 min, and the entire sample electrophoresed in 10% polyacrylamide slab gels with a Laemmli (1970) buffer system. The dried gels were analysed by autoradiography at -70°C for 2–24 h. The assay could detect as little as 0.25 unit of urokinase.

The inhibitor was also tested for stable interaction with urokinase during sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. ^{125}I -urokinase (5 μl) was mixed with 25 μl of test sample made 0.25% with respect to Triton X-100 and incubated for 3 h at 37°C . The sample was then diluted with 25 μl of the electrophoresis buffer, boiled for 2 min, and electrophoresed and analysed by autoradiography as described above.

Protein determination

Protein concentration was determined by the method of Lowry *et al.* (1951), with a bovine albumin standard.

Results

We have previously reported partial purification of an endotoxin-inducible PA inhibitor from mouse peritoneal macrophages. Molecular-sieve chromatography of the cellular lysate yields a single peak of inhibitory activity of approx. 45–50 kDa. This peak has a specific activity some 50–80-fold higher than that of the cell lysate (Chapman *et al.*, 1982). Chromatography of pooled cell-free supernatants of macrophages cultured in the presence of endotoxin also shows a single inhibitor fraction of 45–50 kDa (results not shown). This finding suggests that the inhibitors in the secreted and intracellular compartments may be identical, or nearly so. To test this possibility further, we

determined whether both inhibitor preparations would block the urokinase-mediated conversion of ^{125}I -plasminogen into plasmin. Data in Fig. 1 show that supernatants of cells cultured in endotoxin (lane 3), but not control supernatants (lane 2), block the appearance of the plasmin light chain in the autoradiograph. The slightly increased density of the light chain in lane 2 reflects soluble PA in the medium of endotoxin-free cultures. As expected, lysates of cultured macrophages also blocked the appearance of plasmin in the autoradiograph (lane 5). The number under each lane refers to the percentage inhibition of urokinase activity in a companion fibrin-plate assay. A rough correlation between the inhibition of urokinase activity in the fibrin-plate assay and abrogation of plasmin formation in the plasminogen-conversion array is evident.

Because the secreted and intracellular forms of the PA inhibitor proved to be indistinguishable in size, physical stability (labile at 56°C for 30 min)

Fig. 1. Effects of secreted and intracellular fibrinolytic inhibitors on urokinase-mediated conversion of ^{125}I -plasminogen into ^{125}I -plasmin

^{125}I -plasminogen and urokinase (0.25 unit) were mixed with $15\mu\text{l}$ of: lane 1, Dulbecco's modified Eagle's medium containing 1% (v/v) acid-treated dog serum; lane 2, endotoxin-free cell-free supernatant; lane 3, supernatant from endotoxin-exposed macrophages; lane 4, 0.2% Triton in phosphate-buffered saline; lane 5, lysate of endotoxin-exposed macrophages; lane 6, ^{125}I -plasminogen without urokinase. The H and L refer to the heavy chain and light chain of plasmin respectively. The differences among samples are best visualized as the effect on the appearance of light chain. The numbers below each lane represent mean percentage inhibition of 0.5 unit of urokinase by each sample in a concurrent fibrin-plate assay.

and mechanism of action, we focused further studies on only one form of the inhibitor, the intracellular PA inhibitor. We did this because the cellular lysates consistently had greater inhibitory activity than did the supernatants. Moreover, in the absence of small amounts of serum (1%) in the culture medium, the PA inhibitor accumulated within the cells and much less activity was in the medium (results not shown). The presence of serum, however, interfered with the enzyme assays used. Therefore the studies described below utilized either cellular lysates or a partially purified fraction of the lysates.

Interaction of ^{125}I -urokinase with macrophage PA inhibitor

The autoradiograph shown in Fig. 2 illustrates the interaction of the macrophage PA inhibitor with ^{125}I -urokinase after electrophoresis in sodium dodecyl sulphate/polyacrylamide gels under reduced conditions. Lane 1 shows the ^{125}I -urokinase alone. Lane 2 shows a discrete band at approx.

Fig. 2. Interaction of mouse macrophage and human placental urokinase inhibitors with ^{125}I -urokinase

The Figure depicts an autoradiograph of mixtures of ^{125}I -urokinase and the various inhibitors after gel electrophoresis. Lane 1, heavy chain and light chain of ^{125}I -urokinase alone, apparent size 33 kDa and 18 kDa respectively; lane 2, ^{125}I -urokinase and lysate of inhibitor-rich mouse macrophages; lane 3, ^{125}I -urokinase and lysate of inhibitor-rich macrophages heated at 56°C for 30 min; lane 4, ^{125}I -urokinase and 10 units of placental inhibitor; lane 5, ^{125}I -urokinase and 20 units of placental inhibitor; lane 6, placental inhibitor, heated at 56°C for 30 min.

65kDa after incubation of the enzyme and inhibitor for 60min at 37°C. In multiple other experiments done in both 10% and 7.5% polyacrylamide gels, the complex migrated with apparent molecular mass of 65–67kDa. We also determined the time course of formation of the complex between ^{125}I -urokinase and the inhibitor by terminating the incubation at various times from 5min to 3h at 37°C. A faint band was visible after 5min, and this progressively intensified during incubation up to 1h. After 1h at 37°C, no further change from that shown in lane 2 occurred. We also observed that heating the inhibitor preparation at 56°C for 30min before incubation with urokinase destroyed all binding (lane 3). All functional activity is lost by heating as well. Similarly, preincubation of ^{125}I -urokinase with *p*-nitrophenyl guanidinobenzoate (1mM), which blocks the active site, also totally blocked binding to the PA inhibitor (results not shown).

In addition to the discrete complex at 65–67kDa, there is a broad band of labelled enzyme above the normal migration of the ^{125}I -urokinase heavy chain (33kDa). This broad band was also obliterated by either heating the inhibitor (lane 3) or inactivating the urokinase active site with *p*-

nitrophenyl guanidinobenzoate (results not shown). The autoradiograph in Fig. 2 also shows the interaction of a partially purified preparation of placental inhibitor (lanes 4, 5 and 6). The binding pattern of the placental inhibitor with urokinase appears similar to that of the macrophage inhibitor except that two less dense but discrete bands at 65–67kDa and 60kDa are visible. Increasing the amount of placental inhibitor from 10 to 20 units (lanes 4 and 5) did not change the pattern of interaction. Again, heating the placental inhibitor at 56°C for 30min (lane 6) or inactivation of urokinase with *p*-nitrophenyl guanidinobenzoate blocked all binding.

Fluorimetric assay of the inhibition of urokinase by the macrophage PA inhibitor

The ability of various amounts of the partially purified macrophage PA inhibitor to block urokinase amidolytic activity directly was studied. The inhibitor and enzyme were incubated at 37°C for 60min, the substrate was then added and the initial reaction rate was measured fluorimetrically. Enzymic activity in Fig. 3(a) is expressed as a ratio of the enzyme activity in the presence of inhibitor to that without inhibitor (E/E_0). The data show that

Fig. 3. Inhibition of urokinase amidolytic activity by partially purified mouse PA inhibitor

In (a) various microlitre amounts of inhibitor were incubated for 60min at 37°C with 5.8nM-urokinase. The fluorogenic substrate at final concentrations of 0.1mM (●) or 1.0mM (○) was added and the initial reaction velocity was measured. Enzyme activity is expressed as fractional activity (E/E_0). The line was drawn by linear-regression analysis and the slope then used to calculate concentration of inhibitor present. In (b) various microlitre amounts of a separate inhibitor preparation were mixed with either 5.6nM-urokinase (●) or 11.2nM-urokinase (○) and the assays carried out as in (a).

the fractional inhibition of urokinase activity by increasing amounts of inhibitor was the same in the presence of either 0.1 mM or 1.0 mM concentrations of the substrate. This finding indicates that the inhibition is not reversed by substrate. It should be noted, however, that the fractional inhibition by the inhibitor is independent of substrate concentration only after prolonged incubation of enzyme and inhibitor in the absence of substrate. Like other inhibitors of serine proteinases, the inhibition when enzyme, inhibitor and substrate are mixed together initially (or within 30 min) is competitive (Travis & Salvesen, 1983).

The data in Fig. 3(a) also show that, for fractional inhibition of 0.35 or more, there is a linear decline in urokinase activity with increasing microlitre amounts of inhibitor. Under this condition, the straight line shown in the Figure is defined by the equation:

$$E/E_0 = -(1/E_0) \cdot I_0 + 1$$

where $-1/E_0$ is the slope of the line and I_0 is the concentration of inhibitor added to the reaction. Using this relationship and a known amount of urokinase (5.8 nM) in the reaction, we have calculated the nanomolar concentration of inhibitor in the partially purified preparations. The concentrations in three such preparations were 170 nM (preparation used in Fig. 3a), 112 nM (preparation used in Fig. 3b) and 145 nM. This represents an average of 142 pmol of PA inhibitor recovered from 240×10^6 macrophages.

Data in Fig. 3(b) show that the prediction that the slope of the line should be proportional to enzyme concentration (E_0) can be verified experimentally. In the experiment, either 5.6 nM- or 11.2 nM-urokinase was mixed with various amounts of inhibitor and the initial reaction rate (after 60 min at 37°C) was determined in the presence of 0.1 mM substrate. As judged by the x-axis intercept, a 2-fold increase in nanomolar enzyme concentration resulted in an approximately 2-fold increase in the inhibitor concentration necessary to neutralize the enzyme.

Calculation of kinetic constants for interaction of macrophage and placental PA inhibitors with urokinase

Because the physical binding of the macrophage and placental PA inhibitors to urokinase appears to be quite similar (Fig. 2), we next determined whether the kinetic constants of the two inhibitors were also similar. The association rate constant (k_{on}) and equilibrium dissociation constant (K_i) for the binding of human placental inhibitor and urokinase have been previously reported (Christensen *et al.*, 1982). We had to modify significantly the reaction conditions given in the previous report

because the macrophage preparations contained small amounts of Triton X-100. This markedly influences the reaction rate of urokinase with substrate. By techniques identical with those described in Fig. 3 legend, we determined the nanomolar concentration of inhibitor in the human placental preparations. We then determined the time course of inhibition of 5.8 nM-urokinase by equimolar concentrations of macrophage or placental inhibitor at 37°C at pH 7.4 in the presence of 0.2% Triton X-100. Results are shown in Fig. 4. For each set of data (E/E_0 , t) a value for k_{on} was calculated (Christensen *et al.*, 1982). The mean k_{on} for the macrophage PA inhibitor was $3 \times 10^5 \pm 2 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. This value ($\pm 1 \text{ s.d.}$) represents an average for ten data points and two

Fig. 4. Time course of inhibition of urokinase amidolytic activity by PA inhibitors

Urokinase (5.8 nM) was mixed with equimolar amounts of partially purified macrophage (●) or placental (○) inhibitors in phosphate-buffered saline at 37°C in the presence of 0.2% Triton X-100. After various times, as shown in the Figure, the substrate was added and the initial reaction velocity was measured. Enzyme activity is expressed as fractional activity (E/E_0). The second-order association constants calculated from the data shown for the macrophage and placental inhibitors were $3.0 \times 10^5 \pm 2.0 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $2.8 \times 10^5 \pm 1.2 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively.

different inhibitor preparations. Some lots of macrophage PA inhibitor inexplicably contained less than half the total protein or inhibitor found in the lots used for these experiments. These lots were not used for analysis. As indicated in Fig. 4, the calculated k_{on} for the placental preparations was not significantly different, $2.8 \times 10^5 \pm 1.2 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. Calculated dissociation constants after 1 h incubation at 37°C at equivalent molar amounts of enzyme and inhibitor were $4 \times 10^{-10} \text{ M}$ for both inhibitor preparations.

Discussion

We have previously reported that mouse peritoneal macrophages express an endotoxin-inducible plasminogen-activator inhibitor. In the present study we have extended these observations in several respects. First, the ^{125}I -urokinase binding data indicate that the inhibitor binds to the heavy chain of urokinase. This chain contains the active site; *p*-nitrophenyl guanidinobenzoate blocked the active site and blocked binding. The observed 65–67 kDa complex is smaller than the expected complex of 78–83 kDa (33 kDa heavy chain and 45–50 kDa inhibitor), and this suggests that a fragment of the inhibitor is removed during the interaction with urokinase. Cleavage of an inhibitor by a proteinase during their interaction with subsequent formation of a dodecyl sulphate-stable bond is common among inhibitors of serine proteinases (Travis & Salvesen, 1983). We consider that the broad band above the normal migration position of urokinase in Fig. 2 represents urokinase dissociated from the inhibitor during electrophoresis, for the following reasons. The appearance of the band is blocked by inactivation of either the inhibitor (lane 3) or the enzyme (by *p*-nitrophenyl guanidinobenzoate) before electrophoresis. Also, Golder & Stephens (1983) have identified a urokinase inhibitor in cultured human monocytes. They assayed urokinase activity in gel slices after electrophoresis of mixtures of the monocyte inhibitor and urokinase. They observed a broad band of urokinase activity extending above the normal migration position of uncomplexed urokinase. This would be expected (and compatible with our data in Fig. 2) if enzyme-inhibitor complexes dissociated during the electrophoresis. They did not observe activity at 65–67 kDa, which would also be expected if this represents urokinase complexed with the inhibitor.

Secondly, the kinetic data (Fig. 4) indicate that the inhibitor is fast-acting and progressive in nature. These data also show that the inhibitor resembles the previously characterized human placental inhibitor but not known human serum inhibitors of urokinase. Christensen *et al.* (1982)

reported a second-order rate constant for the placental inhibitor of $3.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. Our data show an association constant for this inhibitor of approximately an order of magnitude lower. The reason for this discrepancy is unclear, but it is probably because of the effect of Triton on the binding characteristics of enzyme, substrate and inhibitor. The addition of 0.2% Triton to the reaction mixture of urokinase and the fluorogenic substrate causes a several-fold increase in the initial reaction velocity (H. A. Chapman, unpublished work). In any case, we could measure no difference in the rate constant between macrophage and placental PA inhibitors (Fig. 4) when both were assayed under identical conditions. In contrast, Waller *et al.* (1983) reported a second-order rate constant of inhibition of urokinase by heparinized human serum of $93 \text{ M}^{-1} \cdot \text{s}^{-1}$. This proved to be due largely to antithrombin III. This value is nearly 10^4 -fold lower than that of either inhibitor studied in the present work. This result indicates the macrophage PA inhibitor is probably not a component of normal human serum. This conclusion is supported by the absence of a dodecyl sulphate-stable complex between urokinase and any serum inhibitor in the 65–72 kDa region in the absence of added heparin (Waller *et al.*, 1983).

Previous reports indicate a number of cell-derived macromolecular proteins capable of directly inhibiting urokinase activity. To our knowledge, none of these inhibitors has been purified to homogeneity. Instead, the inhibitors have been chemically characterized by their ability to form dodecyl sulphate-stable complexes during incubation with ^{125}I -urokinase. Baker *et al.* (1980) reported a 72 kDa complex identified by electrophoresis under reducing conditions of a fibroblast-derived urokinase inhibitor. This inhibitor also interacted strongly with thrombin. Vassalli *et al.* (1984) reported a human monocyte-derived inhibitor, without thrombin-binding properties, that formed a 72 kDa complex with low-molecular-mass ^{125}I -urokinase (33 kDa) under non-reducing conditions. Our observations indicate 60–67 kDa complexes between ^{125}I -urokinase and the macrophage and placental inhibitors. A comparison of the mouse macrophage and human placental inhibitor binding to urokinase (Fig. 2) shows clear differences. However, these may reflect species differences, as the binding patterns of human macrophage and placental inhibitors are virtually indistinguishable (H. A. Chapman & O. L. Stone, unpublished work). A 60–65 kDa complex between the human placental inhibitor and urokinase has been previously reported (Christensen *et al.*, 1982). It is notable that all of these inhibitors are of similar apparent size, 40–50 kDa. These inhibitors may thus represent a group of genetically related

proteins that regulate extracellular PA activity. However, this hypothesis remains to be proven.

The similarities between the macrophage and placental tissue PA inhibitors raise the possibility that placental macrophages are the source of this tissue inhibitor. This is made plausible by the findings that macrophages account for at least 35% of the total cells recoverable from human placenta (Wilson *et al.*, 1983). Interestingly, the macrophage PA inhibitor is not a constitutive cellular protein, but instead is expressed in response to activating stimuli. For example, human alveolar macrophages lavaged from normal lungs do not contain the PA inhibitor. However, these cells express and secrete the inhibitor after stimulation by endotoxin *in vitro* (H. A. Chapman & O. L. Stone, unpublished work). Further study is needed to determine directly whether human placental macrophages produce the PA inhibitor and whether the expression of the inhibitor is subject to regulation by relevant signals, e.g. hormones.

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